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Dedual, Mara A ; Wueest, Stephan ; Borsigova, Marcela ; Konrad, Daniel

Abstract: Four days of high-fat diet (HFD) feeding are sufficient to induce glucose intolerance and hepatic steatosis in mice. While prolonged HFD-induced metabolic complications are partly mediated by increased food intake during the light (inactive) phase, such a link has not yet been established in short-term HFD-fed mice. Herein, we hypothesized that a short bout of HFD desynchronizes feeding behavior, thereby contributing to glucose intolerance and hepatic steatosis. To this end, 12-wk-old C57BL/6J littermates were fed a HFD for 4 days either ad libitum or intermittently. Intermittent-fed mice were fasted for 8 h during their inactive phase. Initiation of HFD led to an immediate increase in food intake already during the first light phase. Moreover, glucose tolerance was significantly impaired in ad libitum- but not in intermittent HFD-fed mice, indicating that desynchronized feeding behavior contributes to short-term HFD-induced glucose intolerance. Of note, overall food intake was similar between the groups, as was body weight. However, intermittent HFD-fed mice revealed higher fat depot weights. Phosphorylation of hormone sensitivity lipase and free fatty acid release from isolated adipocytes were significantly elevated, suggesting increased lipolysis in intermittent HFD-fed mice. Moreover, hepatic mRNA expression of lipogenetic enzymes and liver triglyceride levels were significantly increased in intermittent HFD-fed mice. Importantly, food deprivation decreased respiratory exchange ratio promptly in intermittent- but not in ad libitum HFD-fed mice. In conclusion, retaining a normal feeding pattern prevented HFD-induced impairment of metabolic flexibility in short-term HFD-fed mice.

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Intermittent fasting improves metabolic flexibility in short-term high-fat diet-fed mice

Mara A. Dedual[1,2,3], Stephan Wueest[1,2], Marcela Borsigova[1,2], Daniel Konrad[1,2,3]

[1]Division of Pediatric Endocrinology and Diabetology and [2]Children's Research Center, University Children's Hospital, CH-8032 Zurich, Switzerland

[3]Zurich Center for Integrative Human Physiology, University of Zurich, CH-8057 Zurich, Switzerland

Correspondence to:

Daniel Konrad, MD PhD

University Children's Hospital

Department of Endocrinology and Diabetology

Steinwiesstrasse 75

CH-8032 Zurich

Tel: ++41-44-266 7966; Fax: ++41-44-266 7983

Email: daniel.konrad@kispi.uzh.ch

Abstract

Four days of high-fat diet (HFD) feeding are sufficient to induce glucose intolerance and hepatic steatosis in mice. While prolonged HFD-induced metabolic complications are partly mediated by increased food intake during the light (inactive) phase, such a link has not yet been established in short-term HFD-fed mice. Herein, we hypothesized that a short bout of HFD desynchronizes feeding behavior thereby contributing to glucose intolerance and hepatic steatosis. To this end, 12 week-old C57BL/6J littermates were fed a HFD for four days either ad libitum or intermittently. Intermittent fed mice were fasted for eight hours during their inactive phase. Initiation of HFD led to an immediate increase in food intake already during the first light phase. Moreover, glucose tolerance was significantly impaired in ad libitum but not in intermittent HFD-fed mice, indicating that desynchronized feeding behavior contributes to short-term HFD-induced glucose intolerance. Of note, overall food intake was similar between the groups, as was body weight. However, intermittent HFD-fed mice revealed higher fat depot weights. Phosphorylation of hormone sensitivity lipase (HSL) as well as free fatty acid (FFA) release from isolated adipocytes were significantly elevated suggesting increased lipolysis in intermittent HFD-fed mice. Moreover, hepatic mRNA expression of lipogenetic enzymes as well liver triglyceride levels were significantly increased in intermitted HFD-fed mice. Importantly, food deprivation decreased respiratory exchange ratio promptly in intermittent but not in ad libitum HFD-fed mice. In conclusion, retaining a normal feeding pattern prevented HFD-induced impairment of metabolic flexibility in short-term HFD-fed mice.

Introduction

More than 400 million people worldwide suffered from type 2 diabetes in 2015, with a further increase predicted. Its high prevalence makes it one of the greatest health challenges nowadays (50). In parallel, the worldwide increasing prevalence of obesity, which is the strongest risk factor for insulin resistance and type 2 diabetes, is alarming (25, 59). Therefore, understanding the underlying mechanisms linking obesity to insulin resistance is of utmost interest.

Chronic and short-term high-fat diet (HFD) negatively affect metabolic health (40, 53). Besides food composition and caloric content, timing of food intake plays a pivotal role. Feeding rodents a HFD ad libitum disrupts their diurnal feeding rhythm (23, 32). Under normal chow conditions, mice consume the vast majority of their food during the active/dark phase (14). Of note, a circadian misalignment of food intake and sleep-wake or activity-inactivity rhythm is closely linked to metabolic disease in rodents and humans, and is associated with weight gain, impaired glucose tolerance and/or disturbed lipid metabolism (1, 9, 10, 29, 46). Interestingly, avoiding feeding desynchronization by restricting food access of HFD-fed mice to their active/dark phase prevented HFD-associated adverse metabolic effects (6, 23, 58). Of importance, in some studies the positive impact of prolonged fasting on metabolism in HFD-fed mice, as observed for time-restricted feeding or intermittent fasting regimens, could not be attributed to differences in caloric intake, because cumulative food intake was similar between intermittent fasted and ad libitum-fed animals (23, 38). However, such positive impact of prolonged fasting on metabolism has only been reported in mice after several weeks of HFD-feeding.

We and others have previously shown that glucose tolerance and lipid metabolism were impaired in mice already after a short bout of HFD (8, 19, 22, 27, 34, 53). In the

current study, we aimed to investigate whether a short bout of HFD desynchronizes feeding behavior thereby contributing to glucose intolerance as well as hepatic steatosis and whether re-establishing a diurnal feeding pattern by limiting food access to the active/dark phase prevents metabolic impairments four days HFD-fed mice.

Material and Methods

Animals

12 weeks old male C57BL/6J wildtype littermate mice were assigned to either an ad libitum or an intermittent HFD-feeding regiment for four days (HFD; 59 kcal% fat (coconut oil, mainly saturated fatty acids), 26 kcal% carbohydrates (13% sucrose), 15 kcal% protein; #E15772-347, ssniff-Spezialdiäten GmbH, Soest, Germany). In the intermittent HFD-feeding group, food was removed from the cage for eight hours during the inactive/light phase, whereas ad libitum fed mice had free access to food 24 hours a day. Mice were allocated to groups based on body weight (similar mean +/- SD in starting body weight between the groups).

Mice were housed in a pathogen free animal facility on a 12 h dark/light cycle (light on from 7 am to 7 pm) at room temperature (21°C). After weaning, mice were ad libitum fed a regular chow diet (12 kcal% fat (soybean and sunflower oil, mainly unsaturated fatty acids), 57 kcal% carbohydrates, 31 kcal% protein; #3436, ProvimiKliba, Kaiseraugst, Switzerland) until the age of 12 weeks. The same diet was used for chow-fed mice in experiments. All animal studies were conformed to the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland.

Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance test (ipGTT) was performed in 12 week-old chow-fed mice after five hours of fasting. The test was repeated after one week recovery period followed by the respective four-day feeding-regimen. Glucose (2g/kg body weight) was injected intraperitoneally (ip) and blood glucose concentration was measured from blood received after tail vessel incision after 15, 30, 45, 60, 90 and 120 min by using a glucometer (Accu-Check Aviva, Roche Diagnostics) (33).

Metabolic cage analysis

Food intake and respiratory exchange ratio (RER) were assessed in 24-hour single-housed mice in the PhenoMaster system (TSE Systems, Bad Homburg, Germany). Mice were group-housed before placement in the metabolic cages. One week before starting HFD, mice were single-housed in metabolic cages for two days to adapt and avoid stress during analysis. For data presented in Figure 1, HFD was started immediately after placing mice in metabolic cages. For data presented in Figs. 2 and 5, mice were group-housed for the first 3 days of the feeding regimen before placing them into metabolic cages. Three data points/hour were recorded over a 24 hour period for O_2 consumption and CO_2 production. RER was calculated using the manufacturer's software.

Determination of circulating parameters

EDTA (5 mmol/L) was added to blood collected from the portal vein or heart right after scarification of mice fasted for 5 hours. Blood was immediately centrifuged (4°C, 8000 rpm, 10 min) and obtained plasma was stored at -80°C until further analysis.

Circulating free fatty acid (FFA) concentrations were determined as described using an enzymatic assay (11) (Wako Chemicals GmbH, Neuss, Germany). Insulin was determined as previously described (33).

Weighing and tissue sampling

Ad libitum and intermittent fed mice were weighed daily at the beginning of the fasting period of the intermittent fed mice (at 9 am) and again at the end of the 8-hour fasting period (at 5 pm) using a precision balance (PCP-2000-1, Kern + Sohn, Balingen, Germany). Food was weighed at the same two time points. After the four-day feeding regimen, all mice were fasted at day five from 9.00 am (time of food withdrawal in the intermittent group) to 2.00 pm. Thereafter, mice were euthanized using CO_2 and tissues was collected.

Lipolysis assay

Adipocytes were isolated as previously described (45). 400 µl of packed isolated adipocytes were incubated in 600 µl Krebs Ringer HEPES buffer (KRH) containing 1% (w/v) fatty acid-free BSA (Sigma, Buchs, Switzerland) in the absence or presence of 1µM isoproterenol (Sigma) for one hour. FFA levels in the incubation medium were measured using an enzymatic assay (Wako Chemicals GmbH). Number of incubated cells was determined with a hemocytometer under the light microscope.

Lipid metabolite measurement in liver tissue

1 ml of 50 mM Potassium phosphate buffer was used to lyse 30 mg of frozen liver tissue. Thereafter, lipids were extracted using the protocol of Bligh and Dyer (2) with a

chloroform/methanol solvent (1:2 v/v) containing N-Heptadecanoyl-D-Spingosine and 1,2.Dilaurin (Larodan, Solna, Sweden) as internal standards in a 1 µM final concentration. Lipids were air-dried and redissolved in 200 µl methanol and diluted 5 times in methanol/water (4:1 v/v) containing 10 mM ammonium acetate. Thereafter, lipids were referred to liquid chromatography-mass spectrometry (LC-MS) and analysis was conducted applying the Data Dependent Acquisition (DDA) method. Obtained data were processed and compounds were identified using a data analysis software (ProgenesisQI). Thereafter, identified metabolites were clustered into lipid classes and subclasses (using LIPID MAPS®). Markedly changed metabolites were included into the analysis (≥50% difference in signal intensity between the two groups) and individual thresholds for signal intensity was set for each lipid class to identify the most abundant metabolites within the respective class.

Histology

Epididymal adipose tissue was fixed in 4% buffered formalin and thereafter paraffin-embedded. Embedded tissue was cut (4 µm) and sections were stained with hematoxylin and eosin. Adipocyte perimeter was analyzed in pictures from two different sections per mouse using Image J software (National Institutes of Health, Bethesda, MD, USA) (33).

Triglyceride determination

Triglycerides of about 100 mg liver tissue were extracted according to the method of Bligh and Dyer (2). Extracted triglyceride concentrations were determined using a colorimetric assay (Roche Diagnostics, Rotkreuz, Switzerland).

142

143 *RNA extraction and quantitative reverse transcription-PCR*

144 RNA was extracted with a commercially available kit (NucleoSpin® RNA Set for
145 NucleoZOL, Macherey-Nagel, Düren, Germany) and reverse transcribed as described
146 before (56). To determine relative gene expression levels, cDNA was amplified using
147 TaqMan assay (Applied Biosystems, Foster City, California, USA) and thereafter
148 normalized to 18s RNA using the $2^{-\Delta\Delta CT}$ method (36). The following primers were used:
149 *Acox1* (Mm00443579_m1), *Cpt1* (Mm00550438_m1), *Cd36* (Mm00432403_m1), *Acc1*
150 (Mm01304257_m1), *Fas* (Mm00662319_m1), *Acad1* (Mm00599660_m1), *Pparα*
151 (Mm00627559_m1), *Il6* (Mm00446190_m1), *Il10* (Mm00439614_m1), *Tnfα*
152 (Mm00443258_m1) (Applied Biosystems).

153

154 *Western blotting*

155 Tissue samples were lyzed and protein concentrations were determined as
156 previously described (26). Proteins were separated by sodium dodecyl sulfate
157 polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently, transferred onto
158 nitrocellulose membranes (0.2µm, Bio-Rad, Hercules, California, USA). Membranes were
159 incubated with primary antibodies overnight at 4°C. The following primary antibodies were
160 used: anti-Actin (MAB1501) (Millipore, Billerica, Massachusetts, USA), anti-phospho-ERK
161 1/2 (Thr202/Tyr204, #9101), anti-ERK 1/2, (#9102), anti-phospho-HSL (Ser660, #4216)
162 and anti-HSL (#4107S) (Cell Signaling Technology, Danvers, Massachusetts, USA), anti-
163 PPARγ (H-100) (Santa Cruz Biotechnology, Dallas, Texas, USA).

164

165 *Data analysis*

Data were analyzed by unpaired, two-tailed Student's t-test or, for comparison of ipGTTs before and after feeding the respective regimen, by paired, two-tailed Student's t-test. For multiple group comparison, two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was applied. Data are presented as mean ± SEM. p values < 0.05 were considered significant. Power calculation analysis was not performed. Sample size was determined based on previous experiments performed in our laboratory.

Results

Ad libitum HFD feeding desynchronizes food intake immediately

To investigate whether a short bout of HFD desynchronizes feeding behavior, food intake in 12 weeks old male C57BL/6J wildtype was analyzed in metabolic cages. Immediately before starting analysis, littermates were either assigned to a HFD regimen or were consecutively fed a chow diet. Intake of the respective diet was monitored over 24 hours. Of note, the diet-switch from chow to HFD was conducted during the inactive/light phase. Interestingly, immediately after exposure to HFD, mice began to feed (Fig. 1A). In contrast, littermates remaining under chow diet showed a normal feeding pattern with a clear preference to food intake during the active/dark phase. Consequently, food intake during the inactive/light phase was significantly lower in chow but not in HFD-fed mice (Fig. 1B). Similarly, caloric intake significantly increased in the active/dark when compared to the inactive/light phase only in chow-fed mice (for chow-fed: inactive/light 1.4±0.6 kcal *vs.* active/dark 7.1±1.8 kcal; $p<0.01$) (for HFD-fed: inactive/light 5.2±0.5 kcal *vs.* active/dark 6.5±0.8 kcal; $p>0.9$). HFD-fed mice consumed almost half of their daily food intake during the light phase (Fig. 1C). Of note, the distinct feeding patterns were

also reflected in the respiratory exchange ratio (RER) (Figs. 1D and 1E). As expected, chow-fed mice showed reduced RER during their inactive/light phase, when they abstain from food and therefore switch to lipid oxidation. In contrast, HFD-fed mice, that consumed constantly food, did not show fluctuations in the RER during the course of the day. Taken together, HFD desynchronizes food intake immediately.

Four days of intermittent HFD-feeding imposes a physiological pattern of food intake and fuel utilization

As previously reported by us and other groups, four days of ad libitum HFD-feeding are sufficient to induce hepatic steatosis and impair glucose tolerance (22, 27, 34, 53). Given the fact that HFD feeding immediately affects food intake (Fig. 1A to 1C), we hypothesized that desynchronization in food intake contributes to the observed metabolic alterations in four-day HFD-fed mice. To investigate such hypothesis we assigned 12-week old mice either to a four-day ad libitum HFD-feeding protocol or to a four-day intermittent HFD-feeding protocol (Fig. 2A). Ad libitum fed mice had constant access to food, whereas food access in intermittent fed animals was limited with food deprivation for eight hours during the inactive/light phase. Intermittent fed mice showed a similar four-day cumulative food intake as ad libitum fed littermates (Fig. 2B). Similarly, body weight was not different between the two groups (Fig 2C). However, intermittent fed mice lost more weight during the inactive/light phase and gained more weight during the active/dark phase (Fig. 2D). Daily food intake as shown for the fourth day was similar between the two groups (Figs. 2E and 2F). Whereas RER was similar between the inactive/light and active/dark phase in ad libitum fed mice, RER significantly increased during the active/dark phase in intermittent fed mice (Figs. 2G and 2H). O2 consumption did not

significantly differ between the groups (Fig. 2I), indicating similar energy expenditure. In addition, 24-hour locomotor activity was not different between the groups (ad libitum 2223±37 m *vs*. intermittent 2281±528 m; p=0.9). Collectively, intermittent feeding blunts HFD-induced impairment of metabolic flexibility.

Intermittent HFD-feeding enhances fat accumulation and fasting-induced lipolysis in white adipose tissue

Adipose tissue morphology was assessed next. As depicted in Fig. 3A, weight of epididymal (eWAT), inguinal (iWAT) and mesenteric white adipose tissue (mesWAT) was significantly higher in intermittent HFD-fed compared to ad libitum HFD-fed mice after four days of HFD. Analysis of histological sections revealed increased adipocyte size in intermittent fed mice (Fig. 3B). In order to explore lipolytic activity, phosophorylation of extracellular-signal regulated kinase (ERK) and hormone-sensitive lipase (HSL) was determined in iWAT after five hours of fasting. Phosphorylation of both ERK and HSL were significantly elevated in intermittent compared to ad libitum fed mice (Figs. 3C and 3D). In addition, basal FFA release from isolated epididymal as well as inguinal adipocytes was significantly increased in intermittent compared to ad libitum HFD-fed mice (Figs. 3E and 3F). Of note, isoproterenol-stimulated FFA release was not different between the groups (for epididymal adipocytes: ad libitum 2.2±0.3 µmol/10^6 cells *vs.* intermittent 2.5±0.5 µmol/10^6 cells; p=0.6) (for inguinal adipocytes: ad libitum 3.5±0.4 µmol/10^6 cells *vs.* intermittent 3.3±1.3 µmol/10^6 cells; p=0.9). Furthermore, the finding of increased basal FFA release from adipocytes was accompanied by a ~40% increase of portal free fatty acids (FFA) levels in intermittent fed mice (p=0.12) (Fig. 3G). Taken together, intermittent HFD-feeding enhances adipogenesis and lipolysis.

238

239 *Intermittent HFD promotes hepatic lipid accumulation, β-oxidation and lipogenesis*

240 Liver morphology was determined thereafter. As depicted in Fig. 4A, liver weight
241 did not differ between ad libitum and intermittent fed littermates after four days of HFD. In
242 line with enhanced lipolysis in white adipose tissue, peroxisome proliferator-activated
243 receptor γ (PPARγ) protein levels were increased in the liver of intermittent fed mice (Fig.
244 4B). The latter can be activated upon fatty acid binding and induces a lipogenic program
245 (16). Accordingly, mRNA expression of enzymes involved in lipogenesis was increased
246 (Fig. 4C). The latter was accompanied by a threefold increase in hepatic triglycerides
247 levels (Fig. 4D). Moreover, there was a significant increase in various triglyceride and
248 diacylglycerol fractions in the liver of intermittent compared to ad libitum fed mice (Figs.
249 4E and 4F).

250 PPARα is the major inducer of β-oxidation and similar to PPARγ, it is upregulated
251 in response to fasting (30). As depicted in Fig. 4G, mRNA expression of PPARα as well
252 as of other enzymes involved in β-oxidation was increased in intermittent compared to ad
253 libitum fed mice after four days of HFD. In accordance with an upregulation of β-oxidation
254 enzymes, various fatty acyl carnitine fractions were reduced in intermittent fed mice (Fig.
255 4H). Of note, mRNA expression of the pro-inflammatory cytokines *Tnfα* and *Il6* were
256 reduced in intermittent compared to ad libitum fed mice (Fig. 4I) indicating reduced hepatic
257 inflammation.

258

259 *Four days of intermittent HFD-feeding prevents impairment of HFD-induced glucose*
260 *tolerance and metabolic flexibility*

We next wanted to assess the impact of intermittent HFD-feeding on glucose metabolism. As depicted in Fig. 5A and 5B, glucose tolerance was significantly impaired after four days of ad libitum HFD feeding. In contrast, four days of intermittent HFD did not impair area under the curve (AUC) of a glucose tolerance test (Fig. 5B). When comparing ad libitum *vs*. intermittent HFD-fed mice, there was no significant difference between the two groups (ad libitum 2019±153 mmol/l*min *vs.* intermittent 1807 ± 110 mmol/l*min; p=0.28). Of note, blood glucose disposal curve in intermittent fed mice showed a special pattern with a quick decline 15 minutes after glucose injection and a plateau between 30 and 60 minutes (Fig. 5A). Moreover, there was a trend to increased circulating insulin levels in intermittent HFD-fed mice after 5-hours of fasting (ad libitum 0.94±0.12 ng/ml *vs.* intermittent 1.22±0.10 ng/ml; p=0.10). After four days of HFD-feeding we assessed RER after food deprivation. Importantly, intermittent fed mice depicted a rapid decline in RER immediately after initiation of starvation whereas RER of ad libitum fed mice remained more or less unchanged in the first 60 minutes after food withdrawal (Fig. 5C). Accordingly, mean RER in the first hour after food deprivation was significantly lower in intermittent compared to ad libitum fed mice (Fig. 5D). In contrast, mean O2 consumption did not differ between the groups (ad libitum 4109±206 ml/h*kg *vs*. intermittent 3692±240 ml/h*kg; p=0.25). These results suggest maintained metabolic flexibility in intermittent but not ad libitum HFD-fed mice.

Discussion

The current study suggests that a short bout of HFD desynchronizes feeding pattern as characterized by increased food intake during the inactive/light phase, thereby contributing to glucose intolerance, hepatic steatosis and metabolic inflexibility. Such

notion is based on the following findings: i. a short bout of HFD desynchronizes feeding behavior by increasing food consumption during the inactive/light phase; ii. a short bout of ad libitum HFD impaired glucose tolerance and deprivation-induced decline in RER, which was both prevented in intermittent HFD-fed mice.

Previous studies have shown that limiting food access to the active/dark phase has positive effects on metabolism in rodents and humans (23, 42, 49, 54). Even the time slot during the active/dark phase to which food access is limited to may play an important role in maintaining metabolic health. For instance, adults that skip breakfast are more prone to develop obesity (37). Moreover, food supply in a “breakfast-regimen” with limited food to the beginning of the active/dark phase seems to be beneficial in rodents and in humans (3, 49). In the current study, intermittent-fed mice consumed the vast majority of their daily food during the early active/dark phase, whereas ad libitum fed mice did not exhibit such an increase in food intake in the early active/dark phase. Eating behavior in alignment with the circadian clock might be responsible for the observed beneficial outcome in these time-restricted feeding regimens. Various key-enzymes involved in metabolic processes show very specific fluctuations in activity and expression profile during the course of the day to optimally meet the body’s energetic needs (43). In this context, it has been shown that individuals which a misalignment of eating behavior and sleep/wake-cycle, such as humans suffering from night eating syndrome or shift-workers, had an increased risk to develop metabolic disorders (10, 29, 48). Herein, intermittent HFD feeding, which simulates the feeding pattern of chow fed mice, prevented HFD-induce impairment of glucose tolerance as well as food-deprivation induced rapid decline in RER even though the total amount of food intake was not different. The latter was due to a compensatory increase in food intake during the active/dark phase as was previously reported in mice

that had time restricted access to food (23, 38, 58). Such enhanced food intake during the active/dark phase may explain the observed increase in WAT depot weight, which serves as preventive energy storage.

Fasting-induced increase in FFA levels serve as energy source (7, 15) and, importantly, shifts fuel utilization from glucose to fat oxidation (13, 55). Herein, intermittent HFD-fed mice exhibited increased phosphorylation of ERK 1/2 and HSL in WAT as well as elevated portal FFA levels after five hours of fasting compatible with activated lipolysis (18, 24). Such finding suggests higher FFA flux to the liver in intermittent fed mice, which may be responsible for the observed increase in hepatic triglyceride accumulation. In obese humans, the main source of hepatic triglycerides in the fasted state are adipose-derived FFAs (12). Moreover, lipid infusion in rats has been shown to promote hepatic triglyceride accumulation, indicating that hepatic triglyceride synthesis is dependent on increased FFA flux to the liver (51). In this regard, 24 hours of fasting induced hepatic triglyceride accumulation in C57/BL/6J mice (21). Despite the observed hepatic diacylglycerol and triglyceride accumulation, glucose tolerance was slightly, albeit not significantly, improved intermittent compared to ad libitum HFD-fed mice. What is the potential benefit of increased hepatic triglyceride (and diacylglycerol) storage? Hepatic triglycerides serve as integral part of very low density lipoproteins (VLDL) and are in this form secreted into the circulation in a controlled manner to meet the energetic needs of peripheral tissues (21, 41). Thus, the liver might serve as lipid buffering system to ensure a perpetual fuel supply to peripheral organs in times of starvation. Such function is maintained in intermittent HFD-fed mice, but lost in ad libitum HFD-fed mice. In accordance, time-restricted-fed human subjects were reported to have increased fasting-induced circulating triglycerides levels (49). Moreover, increased triglyceride accumulation

in intermittent HFD-fed mice may be lost over time since previous studies demonstrated diminished hepatic triglyceride in mice that were interval-fasted for a much longer timeframe (5, 6, 23, 54).

In line with enhanced lipolysis and, concomitantly, higher circulating FFA flux, hepatic protein levels of the lipid-responsive PPARγ1 were significantly higher in intermittent HFD-fed mice. PPAR γ1 is a ligand-stimulated transcription factor that can be activated (among others) upon fatty acid-binding resulting in the induction of a lipogenic transcriptional program (17, 47). Of note, mice with a liver-specific loss of PPARγ revealed reduced hepatic triglyceride accumulation, but exhibited impaired glucose tolerance (16, 39). Opposingly, PPARα knockout-mice with liver-specific PPARy1 overexpression depicted enhanced hepatic steatosis (57). Accordingly, hepatic upregulation of PPARy1 in intermittent HFD-fed mice may be responsible for the observed induction of lipogenic target genes such as *Fas* and *Acc1* promoting triglyceride accumulation. In addition, upregulation of PPARy1 may explain reduced expression of pro-inflammatory cytokines as observed in intermittent fed mice (28).

In addition, PPARα, the master regulator of β-oxidation, is upregulated in response to fasting. In contrast to PPARγ, PPARα expression in the liver is not activated by adipose-derived FFA, but shows a strong circadian induction during the fasted/light phase (30, 31, 35). In accordance with such notion, PPARα mRNA expression was increased in intermittent HFD-fed mice. In agreement with PPARα upregulation, mRNA expression of enzymes involved in β-oxidation (*Acad1, Acox1*) were elevated in the liver of intermittent HFD-fed mice. Furthermore, fatty acylcarnitine accumulation was reduced in these mice. Of note, inherited fatty acid oxidation defects lead to an accumulation of fatty acylcarnitines in respect to the underlying mutation (52). In addition, treating HepG2 cells

in vitro with inhibitors of β-oxidation results in fatty acylcarnitine accumulation (20). All these findings therefore suggest a higher degree of β-oxidation in intermittent HFD-fed mice.

Previous studies have shown that intermittent fasting over a longer time period restores fluctuations in fuel utilization and thereby metabolic flexibility (4, 5, 44). Herein we report that already a short bout of HFD for only four days impaired fasting-induced RER decline, which was maintained in intermittent HFD-fed mice. A quick adaptation to changing metabolic demands is the substantial characteristic of metabolic flexibility. During fasting, metabolism switches from a predominantly glucose to a predominantly lipid-based oxidation (19, 20). Our findings herein would suggest that intermittent HFD-fed mice retained a higher degree of metabolic flexibility than ad libitum fed mice: they activated lipolysis in white adipose tissue more efficiently to supply FFA in order to meet peripheral energy requirements. In accordance with such notion, intermittent HFD-fed mice lost significantly more weight during fasting and gained significantly more weight during feeding.

Of note, data in metabolic cages were only recorded during the last 24-hour period of the four-day feeding regimen. While such approach was chosen to allow group-housing of mice during the first three days, recording of metabolic cage data over a longer time period may be beneficial. Moreover, only male mice were included in the present study. It cannot be ruled out that female mice might have behaved differently. While we observed positive effects of short-term intermittent HFD-feeding on metabolism right after finishing the feeding regimen, we cannot speculate on how long such benefits may be maintained. Lipolysis was not assessed by the gold-standard isotope methodology but rather in isolated adipocytes *ex vivo*, which may not always be translated to *in vivo* conditions.

In conclusion, a short bout of HFD desynchronizes feeding behavior and impairs metabolic flexibility, which is prevented by retaining a normal feeding pattern.

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Disclosures

The authors declare no conflict of interests.

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Figure Legends

Fig. 1 Ad libitum HFD feeding desynchronizes food intake immediately

(**A**) Food intake was monitored in metabolic cages during the first day after introduction of HFD (n=5) and compared to chow-fed littermates (n=4). (**B**, **C**) Absolute and proportional food intake as well as changes in RER (**D**, **E**) during active/dark and inactive/light phase (HFD n=5, chow diet n=4). Values are expressed as mean (A, D) or mean±SEM (B, C, E). *p<0.05, **p<0.01, ***p<0.001 (Student's *t*-test (C) or two-way ANOVA (B, E)).

Fig. 2 Four days of intermittent HFD-feeding imposes a physiological pattern of food intake and fuel utilization

(**A**) Study design for intermittent feeding experiment. (**B**) Mean cumulative food intake over four days in ad libitum and intermittent HFD-fed littermates (n=3 per group). Body weight before and after four days of HFD (**C**) and weight changes during active/dark and inactive/light phase over four days of HFD-feeding (**D**) in intermittent and ad libitum HFD-fed mice (n=6 mice per group). Food intake (**E**, **F**), RER (**G**, **H**) and VO_2 (**I**) on the fourth day of the respective feeding-regimen (n=4 ad libitum fed mice, n=3 intermittent fed mice). Values are expressed as mean (E, G) or mean±SEM (B-D, F, H). #p=0.06, *p<0.05 (two-way ANOVA).

Fig. 3 Intermittent HFD-feeding enhances fat accumulation and fasting-induced lipolysis in white adipose tissue

(**A**) Weight of white adipose tissue depots (n=6 mice per group) and (**B**) adipocyte perimeter (n=4 mice per group) of intermittent and ad libitum HFD-fed littermates after five

hours of fasting on the fourth day of the respective feeding-regimen. *Scale bar* represents 100 µm. (**C**, **D**) Representative Western blots and quantification of respective protein levels in white adipose tissue. Protein levels were normalized to ad libitum fed littermates within the same cohort (n=4 mice per group). Basal FFA release from isolated epididymal (**E**) or inguinal (**F**) adipocytes harvested from intermittent (n=4 for epididymal and n=3 for inguinal adipocytes) and ad libitum HFD-fed mice (n=3). (**G**) Portal FFA levels in intermittent and ad libitum HFD-fed mice (n=6 ad libitum fed mice, n=5 intermittent fed mice). Values are expressed as mean±SEM. *p<0.05, **p<0.01 (Student's t-test).

Fig. 4 Intermittent HFD promotes hepatic lipid accumulation, lipogenesis and β-oxidation

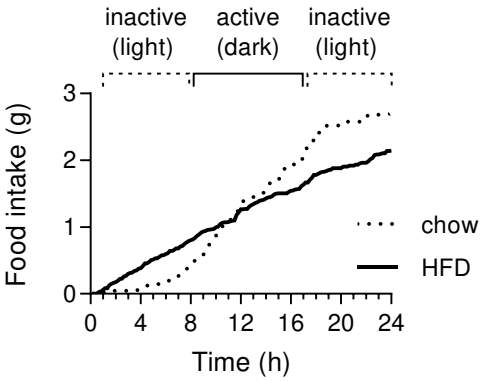
(**A**) Liver weight of intermittent and ad libitum HFD-fed mice (n=6 mice per group). (**B**) Representative Western blot and quantification of PPARγ1 protein levels in liver. Protein levels were normalized to ad libitum fed littermates within the same cohort (n=4 mice per group). (**C**) Relative mRNA expression levels of respective genes (n=4 ad libitum fed mice, n=3 intermittent fed mice). Hepatic triglyceride content (**D**) as well as intensity levels of most abundant TG (**E**) and DG (**F**) are shown (n=4 mice per group). (**G**) Relative mRNA expression of key-enzymes involved in β-oxidation (n=4 ad libitum fed mice, n=3 intermittent fed mice). (**H**) Intensity levels of most abundant fatty acyl-carnitines are depicted (n=4 mice per group). (**I**) Relative mRNA expression levels of pro- and anti-inflammatory cytokines in livers of ad libitum and intermittent HFD-fed mice (n=4 ad libitum fed mice, n=3 intermittent fed mice). Values are expressed as mean±SEM. $^{\#}$p<0.1 (E, F, G), $^{\#}$p=0.07 (I), *p<0.05, **p<0.01, ***p<0.001 (Student's t-test).

Fig. 5 Intermittent HFD prevents HFD-induced impairment of glucose metabolism and metabolic flexibility

(**A**) Intraperitoneal glucose tolerance test (ipGTT) before and after four days of ad libitum or intermittent HFD-feeding (n=9 mice per group). (**B**) Calculation of area under the curve (AUC) for ipGTT presented in Figure A. (**C**, **D**) RER during the first hour of fasting after 4 days of respective HFD-regimen (n=4 ad libitum fed mice, n=3 intermittent fed mice). Values are expressed as mean±SEM. #p=0.06, *p<0.05, **p<0.01 (Student's t-test (A, D) or two-way ANOVA (B, C)).

Figure 1

A



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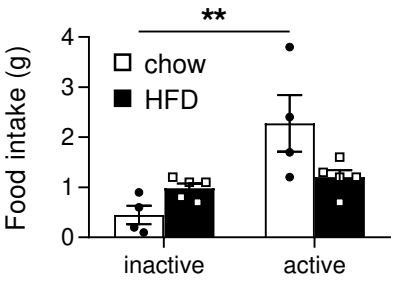

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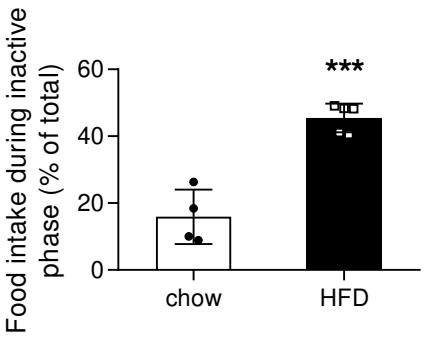

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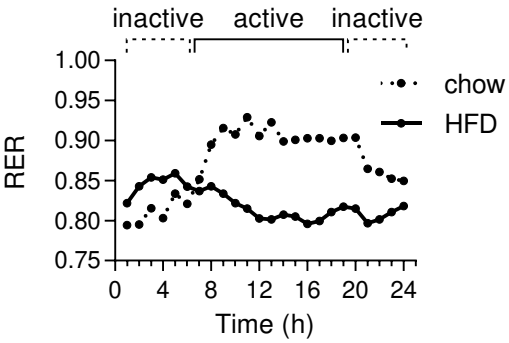

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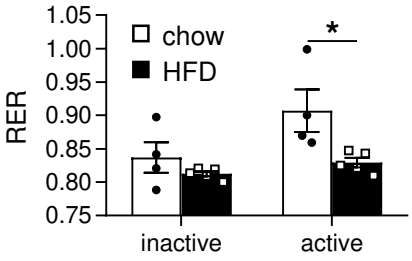


Figure 2

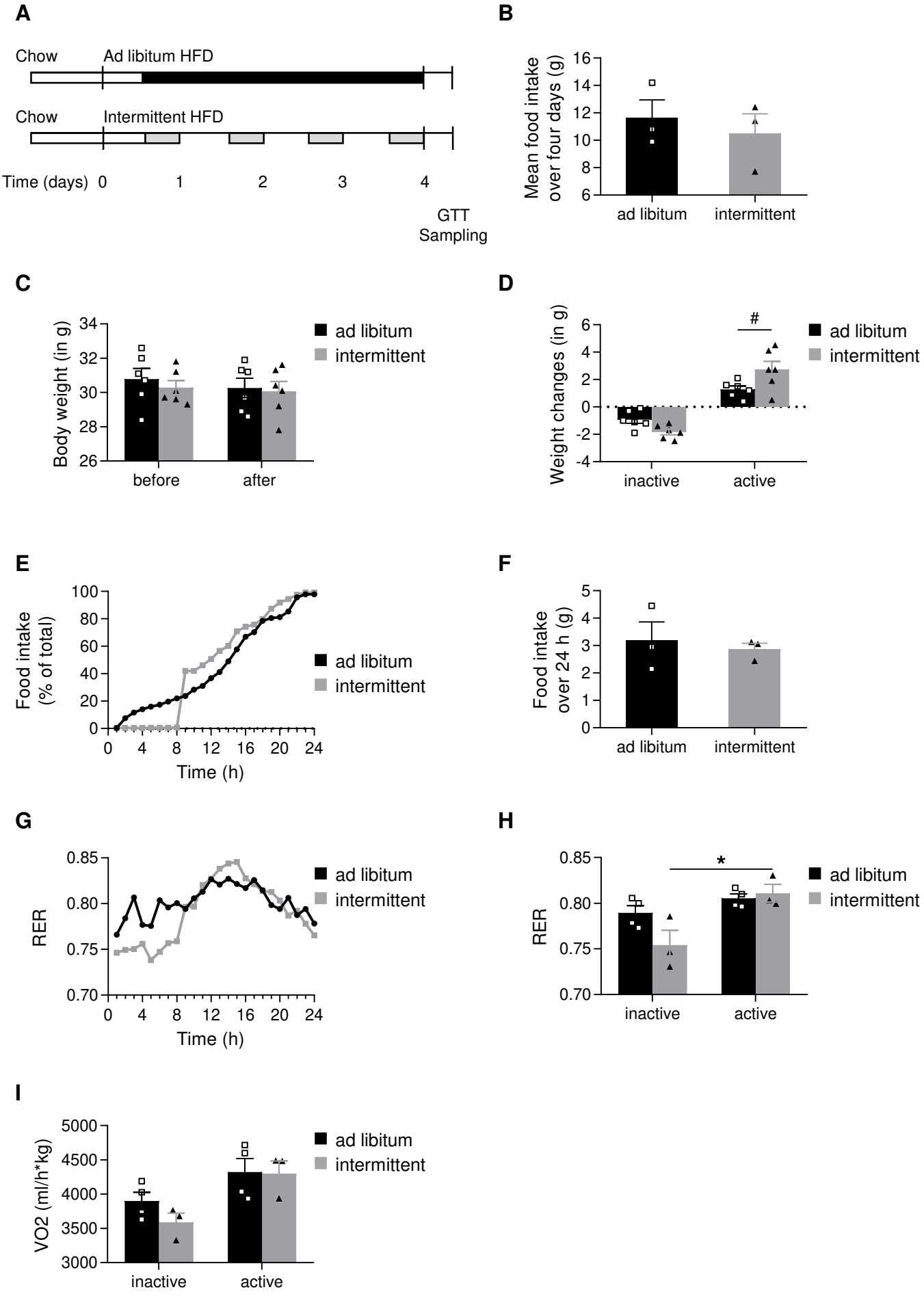

A
Chow
Ad libitum HFD
Chow
Intermittent HFD
Time (days) 0
1
2
3
4
GTT
Sampling
B
Mean food intake over four days (g)
16
14
12
10
8
6
ad libitum
intermittent
C
Body weight (in g)
34
32
30
28
26
before
after
ad libitum
intermittent
D
Weight changes (in g)
6
4
2
0
-2
-4
#
inactive
active
ad libitum
intermittent
E
Food intake (% of total)
100
80
60
40
20
0
0
4
8
12
16
20
24
Time (h)
ad libitum
intermittent
F
Food intake over 24 h (g)
5
4
3
2
1
0
ad libitum
intermittent
G
RER
0.85
0.80
0.75
0.70
0
4
8
12
16
20
24
Time (h)
ad libitum
intermittent
H
RER
0.85
0.80
0.75
0.70
*
inactive
active
ad libitum
intermittent
I
VO2 (ml/h*kg)
5000
4500
4000
3500
3000
inactive
active
ad libitum
intermittent

Figure 3

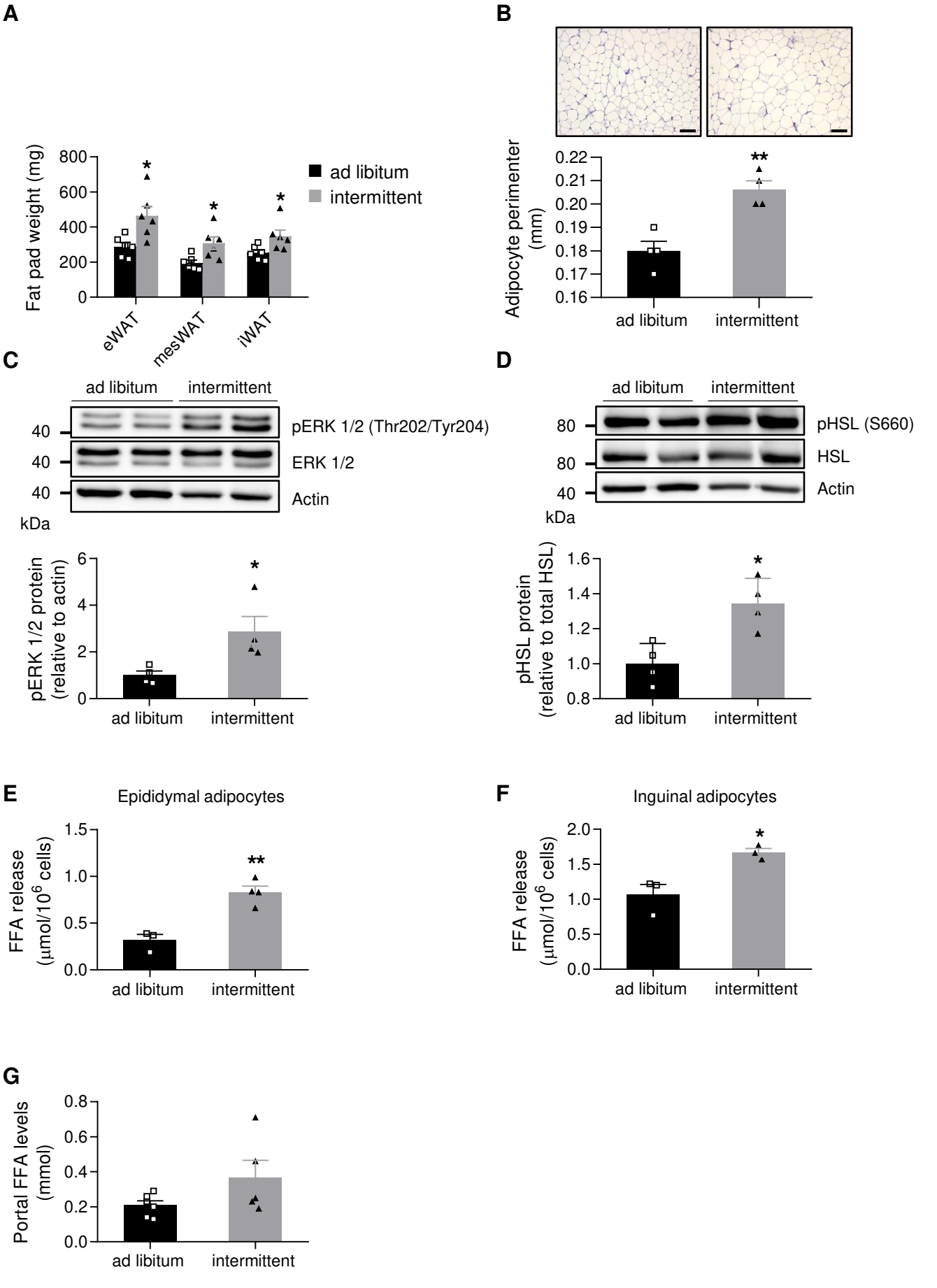
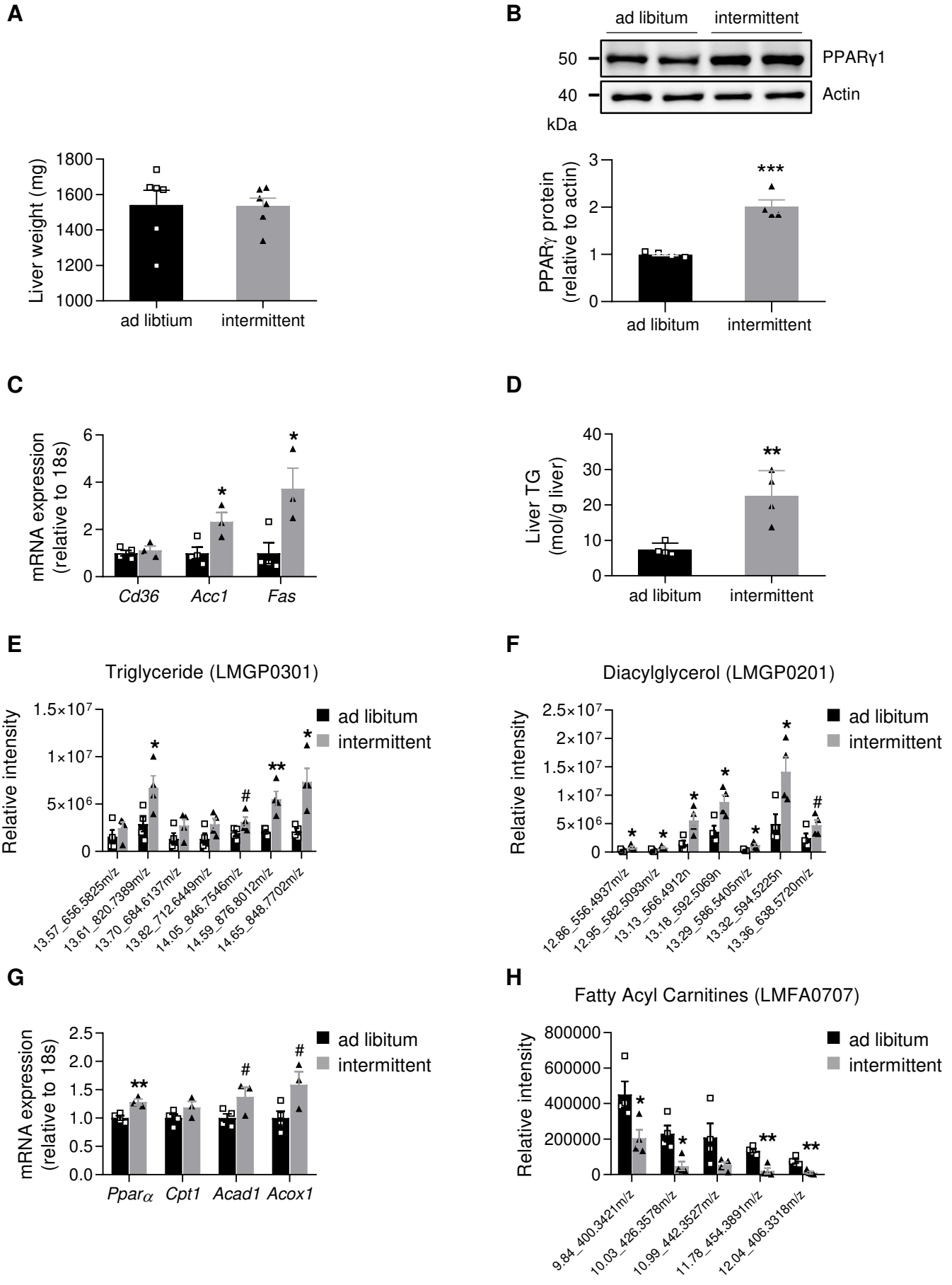

A
Fat pad weight (mg)
800
600
400
200
0
ad libitum
intermittent
eWAT
mesWAT
iWAT
B
Adipocyte perimenter (mm)
0.22
0.21
0.20
0.19
0.18
0.17
0.16
ad libitum
intermittent
C
ad libitum
intermittent
40
40
40
kDa
pERK 1/2 (Thr202/Tyr204)
ERK 1/2
Actin
pERK 1/2 protein (relative to actin)
6
4
2
0
ad libitum
intermittent
D
ad libitum
intermittent
80
80
40
kDa
pHSL (S660)
HSL
Actin
pHSL protein (relative to total HSL)
1.6
1.4
1.2
1.0
0.8
ad libitum
intermittent
E
Epididymal adipocytes
FFA release (µmol/10^6 cells)
1.5
1.0
0.5
0.0
ad libitum
intermittent
F
Inguinal adipocytes
FFA release (µmol/10^6 cells)
2.0
1.5
1.0
0.5
0.0
ad libitum
intermittent
G
Portal FFA levels (mmol)
0.8
0.6
0.4
0.2
0.0
ad libitum
intermittent

Figure 4


A
Liver weight (mg)
1800
1600
1400
1200
1000
ad libtium
intermittent
B
ad libitum
intermittent
50
40
kDa
PPARγ1
Actin
PPARγ protein (relative to actin)
3
2
1
0
***
ad libitum
intermittent
C
mRNA expression (relative to 18s)
6
4
2
0
*
*
*
Cd36
Acc1
Fas
D
Liver TG (mol/g liver)
40
30
20
10
0
**
ad libitum
intermittent
E
Triglyceride (LMGP0301)
Relative intensity
1.5×10^7
1×10^7
5×10^6
0
ad libitum
intermittent
*
*
#
**
*
13.57_656.5825m/z
13.61_820.7389m/z
13.70_684.6137m/z
13.82_712.6449m/z
14.05_846.7546m/z
14.59_876.8012m/z
14.65_848.7702m/z
F
Diacylglycerol (LMGP0201)
Relative intensity
2.5×10^7
2×10^7
1.5×10^7
1×10^7
5×10^6
0
ad libitum
intermittent
*
*
*
*
*
*
#
12.86_556.4937m/z
12.95_582.5093m/z
13.13_566.4912n
13.18_592.5069n
13.29_586.5405m/z
13.32_594.5225n
13.36_638.5720m/z
G
mRNA expression (relative to 18s)
2.5
2.0
1.5
1.0
0.5
0.0
ad libitum
intermittent
**
#
#
Pparα
Cpt1
Acad1
Acox1
H
Fatty Acyl Carnitines (LMFA0707)
Relative intensity
800000
600000
400000
200000
0
ad libitum
intermittent
*
*
**
**
9.84_400.3421m/z
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10.99_442.3527m/z
11.78_454.3891m/z
12.04_406.3318m/z

I

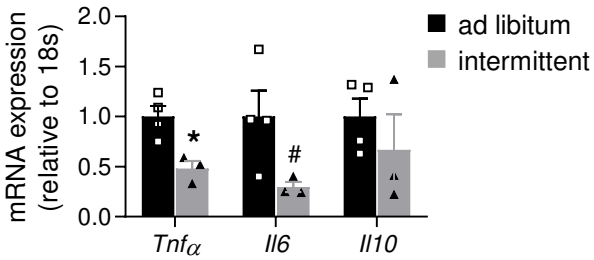
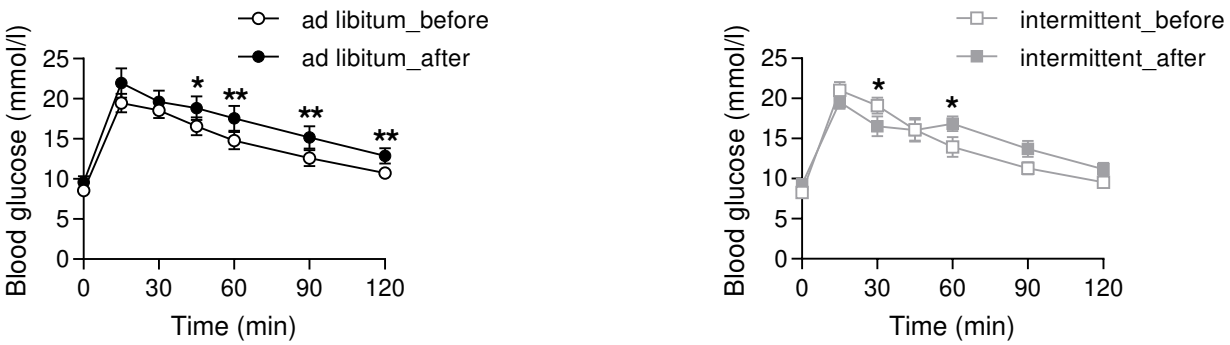

mRNA expression
(relative to 18s)
2.0
1.5
1.0
0.5
0.0
*
#
Tnfα
Il6
Il10
ad libitum
intermittent

Figure 5

A



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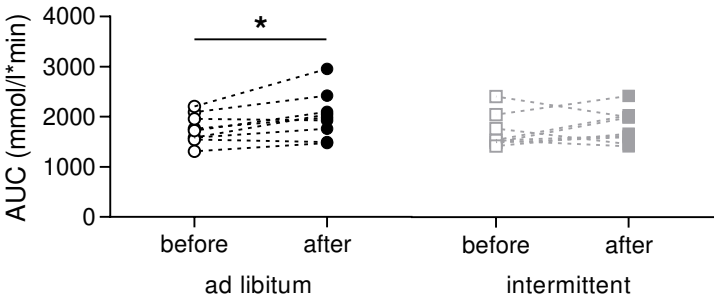

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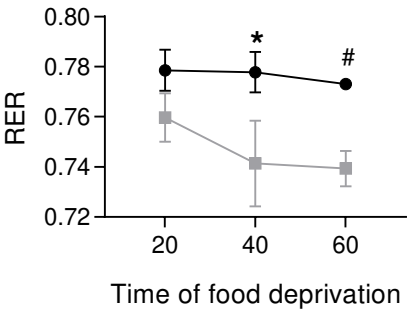

D